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Prevalence and Implication of TT Virus Infection: Minimal Role in Patients With Non-A–E Hepatitis in Taiwan

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TT virus (TTV) is a newly identified human DNA virus and little is known about its clinical significance. The aim of the study was to explore the prevalence of TTV infection in different risk populations and in patients with various liver diseases. Viral DNA was studied in 190 high-risk individuals, 97 household contacts, 52 patients with acute hepatitis A, 32 patients with non-A–E hepatitis including 13 fulminant hepatitis, 200 asymptomatic hepatitis B surface antigen (HBsAg) carriers, 100 patients with chronic hepatitis C, and 100 healthy adults. TTV infection was more frequent in high-risk groups (26–70%), patients with acute or fulminant non-A–E hepatitis (42–45%), and hepatitis C carriers (36%) than in healthy adults (10%) and hepatitis B carriers (15%). However, most of subjects with TTV infection alone had no or only mild hepatitis, and the same rate of TTV DNA in pre-hepatitis serum samples and constant serum TTV titers during hepatitis episodes were observed in two patients with acute non-A–E hepatitis. Phylogenetic analysis of the Taiwanese TTV isolates showed genetic heterogeneity and most (68%) isolates were TTV type 1. No particular strain was found to be associated with fulminant non-A–E hepatitis. ***J. Med. Virol. 59:307–312, 1999.***

KEY WORDS: TT virus; non-A–E hepatitis; Taiwanese TTV isolates

INTRODUCTION

Chronic liver disease and hepatocellular carcinoma are endemic in Taiwan, and most cases can be attributed to hepatitis B virus (HBV) or hepatitis C virus (HCV) infection [Chen, 1987; Chen et al., 1990]. However, there still remains a proportion of hepatitis cases with undefined etiology, suggestive of the existence of additional causative agents [Alter 1994; Kao et al., 1996b]. Although a transfusion-transmissible flavivirus-like RNA virus, GB virus-C (GBV-C) or hepatitis G virus (HGV) [Simons et al., 1995; Linnen et al., 1996], has been claimed to be associated with fulminant and chronic hepatitis in earlier reports [Yoshiba et al., 1995], most recent studies indicate that GBV-C does not cause liver disease [Berenguer et al., 1996; Kao et al., 1996a, 1997b; Wang et al., 1996; Alter et al., 1997; Laskus et al., 1997].

In 1997, a new DNA virus was isolated from a patient with posttransfusion hepatitis of unknown etiology and designated TT virus (TTV) for the initials of the index patient [Nishizawa et al., 1997]. In addition, TTV genomes were detected in patients with cryptogenic posttransfusion hepatitis and the emergence of viremia coincided with the modest increases of serum alanine aminotransferase (ALT) levels [Okamoto et al., 1998b]. Further molecular studies showed that TTV shares several characteristics with parvovirus, such as high buoyant density and a single-stranded, unenveloped, linear DNA genome of at least 3,700 bases [Okamoto et al., 1998b]. In contrast, the complete nucleotide sequence of TTV has been determined recently [Mushahwar et al., 1999], who found that TTV genome is circular and negative stranded, and comprises 3,852 bases with a particle size of 30–50 nm. These results suggest that TTV is similar to the *Circoviridae*. TTV has a wide range of sequence divergence, allowing classification into genotypes (1 and 2) differing by approxi-

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mately 30%, each of which divide into subtypes (a and b) differing by approximately 15% [Okamoto et al., 1998b]. In Japan, TTV DNA was detected in 12% of healthy blood donors, 47% of patients with fulminant non-A–E hepatitis, and 46% of patients with chronic liver diseases of unknown etiology [Okamoto et al., 1998b], suggesting that TTV may be the cause of some cryptogenic liver diseases. However, the clinical significance of TTV infection remains controversial based on the results of several studies [Charlton et al., 1998; Naoumov et al., 1998; Simmonds et al., 1998]. Thus the association of TTV infection with liver cell damage is far from clear, and further studies are needed to investigate the clinical implications of TTV infection. Taking advantage of the extremely common chronic HBV and HCV infections in Taiwan, the presence of TTV viremia was investigated in various populations at risk for parenteral viral infection and patients with different categories of liver disease. The possible role of TTV infection in non-A–E hepatitis was investigated in those with fulminant hepatic failure.

MATERIALS AND METHODS

Subjects

Serum samples were studied from 771 patients and subjects divided into 10 groups. These included (i) 50 illicit intravenous drug users; (ii) 50 hemophiliacs; (iii) 40 polytransfused thalassemic patients; (iv) 50 uremic patients on maintenance hemodialysis; (v) 97 household contacts of TTV-infected patients, including 40 spouses and 57 nonspousal family members; (vi) 52 patients with acute hepatitis A defined by IgM hepatitis A antibody (IgM anti-HAV); (vii) 30 patients with non-A–E hepatitis (acute hepatitis in 12, chronic hepatitis in 9, and fulminant hepatic failure in 11) defined as negative for known serological markers including IgM anti-HAV, IgM anti-HBc, hepatitis B surface antigen (HBsAg), anti-HCV, anti-HDV, and anti-HEV as well as absence of hepatitis viral genomes including HBV DNA, HCV RNA, HDV RNA, HEV RNA, and GBV-C/HGV RNA by nucleic acid amplification methods. The patients had no clinical evidence of fatty liver, drug-induced liver injury, alcoholism, or autoimmune hepatitis and IgM antibodies to Epstein-Barr virus and cytomegalovirus were not detected; (viii) 200 asymptomatic HBsAg carriers with persistence of HBsAg and normal serum ALT levels for at least 1 year; (ix) 100 chronic hepatitis C patients with anti-HCV and serum HCV RNA; and (x) 100 healthy adults who had no risk for hepatitis, normal serum ALT levels, and who were negative for serological markers of current hepatitis viral infection. Acute hepatitis was defined as symptomatic hepatitis with elevated serum ALT levels more than 10 times of upper normal limit (31 U/L) in the absence of a past history of hepatitis. Fulminant hepatic failure was defined as occurrence of encephalopathy within 8 weeks after onset of jaundice in the absence of preexisting liver disease. The diagnosis of chronic liver disease was based on generally accepted clinical and pathologic grounds [International Group, 1977]. Those individuals with dual infections by HBV and HCV were excluded. Serum samples from each subject were stored at −70°C until use.

Serological Markers

IgM anti-HAV, IgM anti-HBc, HBsAg, anti-HCV, anti-HDV and anti-HEV were tested with commercially available kits (HAVAB-M, CORAB-M, Ausria II, HCV EIA II, Anti-Delta, Abbott Laboratories, North Chicago, IL and Genelabs and Diagnostic Technology, Singapore).

Amplification of Hepatitis Viral Genomes

Serum HBV DNA was detected by a polymerase chain reaction (PCR) assay using primers from the core region [Kao et al., 1998]. HCV RNA was assayed by reverse transcription (RT)-PCR with nested primers from the most conserved 5′-untranslated region (5′UTR) of the viral genome [Kao et al., 1992]. HDV and HEV RNA were detected with RT-PCR as previously reported [Reyes et al., 1990; Chao et al., 1991]. GBV-C/HGV RNA was detected by RT-PCR with primers from the 5′-untranslated region of the viral genome [Kao et al., 1997a]. To avoid false-positive results, instructions to prevent cross contamination were followed strictly [Kwok and Higuchi, 1989], and results were considered valid only when they were obtained consistently in at least two separate runs.

Amplification, Titration, and Sequencing of TTV DNA

The presence of TTV DNA was assayed by nested PCR with primer pairs from the open reading frame (ORF)-1 of the viral genome. The oligonucleotide primers were synthesized based on the published TTV sequences [Okamoto et al., 1998b]. Briefly, total DNA was extracted from 100 μl serum using QIAamp Blood kit (QIAGEN Ltd, Crawley, UK) and resuspended in 50 μl elution buffer. For the first stage PCR, 25 μl of reaction mixture containing 2 μl of the cDNA sample, 1× PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, and 0.1% Triton X-100), 10 mM of each dNTP, 100 ng of each outer primer (outer sense: T-1s 5′-ACAGACAGAGGAGAAGGCAACATG-3′; outer antisense: T-2a 5′-CTACCTCCTGGCATTT-TACC-3′), and 1 unit of Taq DNA polymerase were amplified in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) for 30 cycles. Each cycle entailed denaturation at 95°C for 60 sec, primer annealing at 55°C for 30 sec and extension at 72°C for 60 sec with a final extension step at 72°C for 7 min. After the first amplification, 1 μl of the PCR products was reamplified for another 30 cycles with 100 ng of each inner primer (inner sense: T-3s 5′-GGCAACATGTTATGGATAGAC-TGG-3′; inner antisense: T-4a 5′-CTGGCATTTTAC-CATTTCCAAAGTT-3′). The second round of PCR was done in the same manner as the first round giving a 272-bp amplification product. The amplified products were separated by 3% agarose gel electrophoresis and stained by ethidium bromide. Nucleotide sequences of selected amplified products were determined directly

TABLE I. Prevalence of Serum TT Virus DNA in Different Subjects in Taiwan

Subjects	No. studied	TTV DNA No. positive	TTV DNA %
Intravenous drug users	50	14	28*
Hemophiliacs	50	35	70**
Thalassemics	40	27	67.5**
Hemodialysis patients	50	13	26***
Household contacts			
Spouse	40	3	7.5
Nonspouse	57	7	12.3
Acute hepatitis A	52	4	7.7
Non-A–E hepatitis			
Acute	12	5	41.6*
Chronic	9	2	22.2
Fulminant	11	5	45.4*
Hepatitis B carriers	200	30	15
Hepatitis C carriers	100	36	36**
Healthy adults	100	10	10

*$P < .01$, **$P < .001$, ***$P < .05$ by Chi-square test with Yates' correction or Fisher's exact test.

by using fluorescence-labeled primers with a 373A Sequencer (Applied Biosystems, Foster City, CA) to verify the specificity. Sequencing conditions were specified in the protocol for the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). The inner primer pair T-3s and T-4a was used as sequencing primers for both directions of the partial ORF-1 of TTV genome.

Phylogenetic Analysis

A phylogenetic tree was constructed by using the program of unweighted pair-group method with arithmetic mean (UPGMA) method (PHYLIP [Phylogeny Inference Package], version 3.5c; J. Felstein, University of Washington, Seattle) based on the nucleotide sequences (positions 1960–2180) of the amplified ORF-1 of TTV genome. The dataset was bootstrap resampled 100 times to ascertain support for major branches of the tree.

Statistical Analysis

Data were analyzed by Fisher's exact test, Chi-square test with Yates' correction or Student's *t*-test where appropriate. A *P* value of less than .05 was considered significant.

RESULTS

The prevalence of TTV viremia was significantly higher in at risk groups including intravenous drug users, hemophiliacs, thalassemic patients, and patients on maintenance hemodialysis than that in general adult population (26–70% vs. 10%, $P < .05$, Table I). In contrast, the household contacts (spouse and nonspouse family members) of TTV-infected patients had a similar rate of TTV DNA positivity to that of healthy adult controls. Further analysis of the stored serial serum samples (available in three positive hemophiliacs) found that TTV viremia could persist for longer than 3 years in two samples, and another sample had transient viremia. The association between TTV and hepatitis was difficult to analyze in intravenous drug users and hemophiliacs, as most of these subjects were also infected with HCV. Among 24 thalassemic patients with TTV infection alone, 18 (75%) had normal serum ALT levels and the remaining 6 had slightly elevated serum ALT levels (42–82 U/L). In the two viremic chronic non-A–E hepatitis patients, although both had fluctuating serum ALT levels for more than 3 years, the peak levels never exceeded fivefold of the upper limit of normal.

Among patients with various liver diseases, the prevalence of TTV infection was significantly greater in those with acute or fulminant non-A–E hepatitis and chronic hepatitis C than that in general population (36–45% vs. 10%, $P < .01$, Table I). However, the prevalence was comparable among acute hepatitis A patients, chronic non-A–E hepatitis patients, asymptomatic HBsAg carriers and healthy adults. Of these 82 patients positive for TTV viremia, 16 (20%) had a past history of blood transfusion and 52 (63%) were men.

Of 10 TTV DNA-positive patients with acute or fulminant non-A–E hepatitis, none had received blood or blood products previously. A mortality of 80% was observed in those with TTV infection and fulminant hepatic failure. Prehepatitis serum samples were available in 2 patients with acute non-A–E hepatitis, and all were already positive for TTV DNA by PCR. In the first patient, all but one serum sample following onset of hepatitis were positive for TTV DNA, and the viremia persisted for more than 30 months. A similar situation was observed in the second patient and the viremia had been present for longer than 16 months. Serum TTV DNA levels appeared constant (10^1 titer/ml) during hepatitis episodes in both patients and remained unchanged (10^1–10^2 titer/ml) during the follow-up periods.

TTV DNA genomes from 12 patients with non-A–E hepatitis including 5 with acute hepatitis, 2 with chronic hepatitis, and 5 with fulminant hepatitis as well as 10 healthy adults were sequenced directly. Phylogenetic analysis of nucleotide sequences from the amplified ORF-1 of viral genome (positions 1960–2180) and isolates reported previously (TTV type 1a, 1b, 2a and 2b) from Japan was also carried out. The results showed genetic heterogeneity among the 22 isolates cloned from Taiwan, and 9 isolates (41%) were related more closely to the TTV type 1a isolate and 6 (27%) were genetically similar to the TTV type 1b isolate (Fig. 1). In addition, 3 (14%) and 1 (5%) isolates were related to the TTV type 2a and 2b, respectively. The remaining 1 (CH-2) and 2 (AH-2 and NC-6) Taiwanese isolates were designated provisionally as TTV type 1c and 2c, respectively. Thus most (68%) of the Taiwanese TTV isolates were deduced to be TTV type 1. Meantime, we could not find any particular strain that was associated with acute, chronic, or fulminant non-A–E hepatitis.

DISCUSSION

By using representational difference analysis, a novel human virus named TTV in association with hepatitis flares was isolated from a patient with posttransfusion hepatitis of unknown etiology [Nishizawa et al., 1997]. TTV may replicate in liver cells, because its DNA is detected in the liver in titers from 10 to 100 times higher than in the corresponding serum from some patients with chronic non-A–E hepatitis [Okamoto et al., 1998b]. In addition, its serum titer may rise and fall in parallel with serum ALT levels, and become undetectable in patients whose aminotransferases become normal [Okamoto et al., 1998b]. These characteristics make TTV a relatively attractive candidate virus as a potential cause of liver disease. Neither of these characteristics, hepatotropism or correlation of viral titers with serum ALT levels, were ever demonstrated for GBV-C/HGV [Berenguer et al., 1996; Wang et al., 1996; Alter et al., 1997; Kao et al., 1997b; Laskus et al., 1997].

Although reliable serologic assays are not yet available, previous studies based on diagnostic PCR procedures to detect TTV DNA in plasma or serum samples from different populations have shown that the virus can be transfusion transmissible, distributed widely, and can induce persistent viremia in humans [Okamoto et al., 1998b; Simmonds et al., 1998]. In general, TTV is common in populations at risk of infection with blood-borne viruses, such as hemophiliacs (68%) or patients on maintenance hemodialysis (46%), and abusers of intravenous drugs (40%) [Okamoto et al., 1998b]. However, caution must be taken in interpreting these results, because the PCR assay used currently detects viremia only; those who recovered from the infection cannot be detected, as in the situation of earlier epidemiological studies of GBV-C/HGV infection [Simons et al., 1995; Yoshiba et al., 1995; Kao et al., 1996a, 1997b; Linnen et al., 1996; Wang et al., 1996; Alter et al., 1997]. Meantime, the much higher rate of TTV viremia compared with that of GBV-C/HGV in similar at risk populations also suggests that TTV infection may have a greater opportunity of chronicity; however, long-term prospective studies for blood-transmitted diseases are needed to confirm this speculation.

By using a similar PCR method, it was found that the TTV DNA-positive rate of healthy Taiwanese adults was 10%, comparable to that of blood donors in Japan (12%) and healthy control in United Kingdom (10%) but apparently higher than that of blood donors in United States (1%) and United Kingdom (1.9%) [Charlton et al., 1998; Naoumov et al., 1998; Okamoto et al., 1998b; Simmonds et al., 1998]. A significantly increased prevalence was demonstrated by the present study in the parenteral drug users (28%), hemophiliacs (70%), thalassemics (67.5%), and patients on maintenance hemodialysis (26%) who were exposed frequently to blood and blood products (Table I), confirming the importance of the parenteral route of its transmission. Moreover, the existence of persistent TTV viremia was documented in several infected patients.

Many hepatitis viruses share the same modes of transmission, thus multiple viral infection may occur in a given patient [Pontisso et al., 1993]. Co-infection of TTV has been observed frequently in patients with chronic hepatitis B and C [Naoumov et al., 1998]. Taking advantage of the extremely common chronic HBV and HCV infections in Taiwan, the presence of TTV co-infection was investigated in HBV and HCV carriers. Our results showed consistently a significantly higher prevalence of TTV infection in patients with chronic hepatitis C (36%) than in healthy adults (10%) (Table I), implying that HCV and TTV may share common modes of transmission. By contrast, the prevalence in asymptomatic HBsAg carriers was similar to that in the general population (15% vs. 10%) (Table I). These findings are not unexpected because most HBsAg carriers in Taiwan contracted HBV infection during the perinatal period or early childhood [Chen, 1987], and superinfection of TTV may occur thereafter.

Fecal excretion of TTV has been shown in patients with posttransfusion non-A–E hepatitis [Okamoto et al., 1998a], and the possibility of nonparenteral infection by the fecal-oral route has been raised. However, the TTV viremic rate did not increase in the household contacts including spouse and nonspouse family members of TTV-infected patients and the patients with acute hepatitis A virus infection, compared with that of healthy adult controls (Table I). Although these data argue against the efficiency of nonparenteral TTV infection such as through sexual contact or fecal-oral transmission, further large-scale epidemiological studies are needed to resolve this interesting issue.

The clinical implications of TTV infection remain unclear [Charlton et al., 1998; Naoumov et al., 1998; Okamoto et al., 1998b; Simmonds et al., 1998]. In our limited number of thalassemic patients with chronic TTV infection alone, most had no or only mild elevation of serum ALT levels. In addition, our long-term prospective study for transfusion-transmitted diseases also showed that although TTV can be transmitted by transfusion in some patients who underwent cardiac surgery, TTV does not seem to cause hepatitis in most instances (Wang JT et al., unpublished results). Taking these data together, TTV does not cause significant liver injury as classical hepatitis viruses. Further animal and human studies are thus needed to determine the transmissibility and pathogenicity of TTV infection, as well as the association of this common new agent with idiopathic hepatitis and other diseases.

Although in the current study the prevalence of TTV infection was significantly higher in patients with acute or fulminant non-A–E hepatitis (42–45%) than in healthy controls (10%) (Table I), this result did not necessarily imply a causative role for TTV. The higher prevalence of TTV infection in such patients may simply reflect higher rates of parenteral and nonparenteral exposure to TTV than was experienced by the healthy controls. The positivity of TTV DNA in pre-

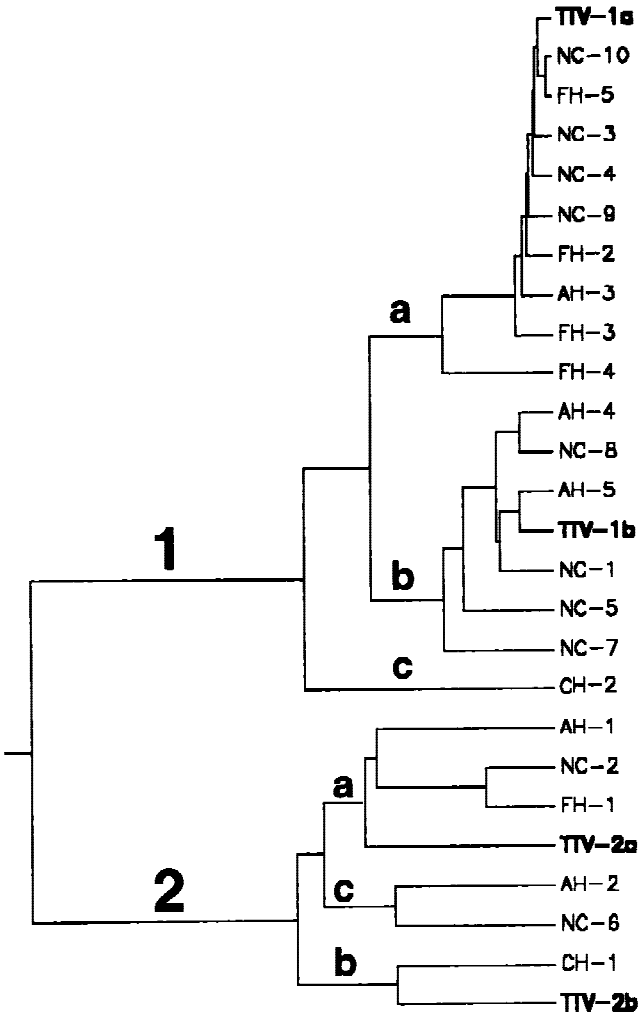

Fig. 1. Phylogenetic analysis of TT virus (TTV) including the original clone of genotype 1a, one each of clones of genotype 1b, 2a, and 2b reported from Japan [Okamoto et al., 1998b], as well as 22 Taiwanese isolates cloned from normal controls (NC), patients with acute (AH), chronic (CH) or fulminant (FH) non-A–E hepatitis based on 221-bp sequences (nucleotide positions 1960–2180) of the open reading frame (ORF)-1. The phylogenetic tree was constructed by the program of unweighted pair-group method with arithmetic mean (UPGMA) method (PHYLIP (Phylogeny Inference Package), version 3.5c; J. Felstein, University of Washington, Seattle). Note the diverse heterogeneity and genotype deduction of the 22 Taiwanese TTV isolates.

hepatitis serum samples and the constant serum TTV titers during hepatitis episodes in our patients with acute non-A–E hepatitis suggested that TTV was unlikely to be the etiological agent of hepatitis in these patients. However, the possibility that in a subset of TTV-infected patients, TTV may cause acute or chronic liver injury because of host or viral characteristics such as genotype variations cannot be excluded.

Genetic variants of TTV have been identified in previous studies [Naoumov et al., 1998; Okamoto et al., 1998b; Simmonds et al., 1998]; however, an association between infection with some particular strains of TTV and the occurrence of hepatitis remains virtually unknown. In the present study, phylogenetic analysis of nucleotide sequences from the ORF-1 of TTV genome showed that genetic heterogeneity of isolates cloned from Taiwan indeed existed, and most (68%) of the Taiwanese TTV isolates were TTV type 1 (Fig. 1). The distribution of TTV genotypes in Taiwan is therefore similar to that in Japan, and the close relationships between Taiwanese and Japanese strains of TTV can be explained historically and geographically, as in the situation of HCV, HDV, and GBV-C/HGV [Kao et al., 1995; Wu et al., 1998]. Importantly, the phylogenetic analysis showed no evidence of the association of non-A–E hepatitis with any particular strain of TTV. This fact argues strongly against TTV being related to acute or fulminant hepatitis by itself.

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